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A Dynamically Localized Protease Complex and a Polar Specificity Factor Control a Cell Cycle Master Regulator

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SUMMARY

Regulated proteolysis is essential for cell cycle progression in both prokaryotes and eukaryotes. We show here that the ClpXP protease, responsible for the degradation of multiple bacterial proteins, is dynamically localized to specific cellular positions in *Caulobacter* where it degrades colocalized proteins. The CtrA cell cycle master regulator, that must be cleared from the *Caulobacter* cell to allow the initiation of chromosome replication, interacts with the ClpXP protease at the cell pole where it is degraded. We have identified a novel, conserved protein, RcdA, that forms a complex with CtrA and ClpX in the cell. RcdA is required for CtrA polar localization and degradation by ClpXP. The localization pattern of RcdA is coincident with and dependent upon ClpX localization. Thus, a dynamically localized ClpXP proteolysis complex in concert with a cytoplasmic factor provides temporal and spatial specificity to protein degradation during a bacterial cell cycle.

INTRODUCTION

Regulated proteolysis plays a critical role in controlling cell cycle progression (Domian et al., 1997) and cell polarity (Chen et al., 2005; Viollier et al., 2002) in *Caulobacter crescentus*, competence and sporulation in *Bacillus subtilis* (Msadek et al., 1998), and stationary phase survival and stress response in *E. coli* (Alba et al., 2002; Lange and Hengge-Aronis, 1994). In each of these cases, a critical regulatory factor or determinant undergoes controlled, specific proteolysis in response to environmental or intracellular signals. Targeted proteolysis in bacteria employs multisubstrate proteases such as ClpXP, ClpAP, and HslUV, with functional and structural similarity to the 26S proteasome (Gottesman, 2003). These proteases are multisubunit complexes with a guarded active site into which entry is controlled by a separate ATPase domain. The ATPase component is responsible for substrate selection and supplies the energy for unfolding and translocating the substrate into the proteolytic chamber. In the few well-characterized cases of regulated proteolysis in bacteria, factors have been identified that enhance or are necessary for the targeted degradation of proteins by a multisubstrate ATP-dependent protease (Ades, 2004; Gottesman, 2003).

Temporally regulated proteolysis plays a significant role in controlling cell cycle progression in *Caulobacter crescentus* (Crosson et al., 2004; Skerker and Laub, 2004; Viollier and Shapiro, 2004). *Caulobacter's* cell cycle (Figure 4A) includes an asymmetric cell division that results in progeny of different cell types—a swarmer cell and a stalked cell. The swarmer cell, which is equivalent to the G1 phase of the eukaryotic cell cycle, contains a single quiescent chromosome and can be morphologically distinguished from other cell stages by its smaller size, single polar flagellum, multiple polar pili, and absence of the stalk. Initiation of S phase, that is concurrent with the morphological transformation of a swarmer cell to stalked cell, results in a single round of DNA replication. Subsequent cell division results in two cells of distinct fates—a stalked cell immediately able to initiate DNA replication and a replication-quiescent swarmer cell.

Differential activity of the essential cell cycle master regulator CtrA is critical to control of the G1-S transition. CtrA is a DNA binding response regulator whose activity is controlled by phosphorylation on aspartate 51 (D51) in its receiver domain. *Caulobacter* cell cycle progression requires cyclical presence of activated CtrA (Domian et al., 1997; Quon et al., 1996). CtrA is redundantly controlled at the levels of transcription, phosphorylation state, proteolysis, and cellular localization (Holtzendorff et al., 2004; Hung

and Shapiro, 2002; Jacobs et al., 1999; Reisenauer and Shapiro, 2002; Ryan et al., 2004). CtrA directly controls the transcription of almost 100 genes, and it also prevents replication initiation in swarmer cells by binding to five sites in the origin of replication (Laub et al., 2002; Quon et al., 1996, 1998). DNA replication can only begin when CtrA activity is eliminated from the cell (Domian et al., 1997).

Activated CtrA is removed from the cell by the redundant mechanisms of temporally controlled dephosphorylation and proteolysis. Only if both of these CtrA control mechanisms are blocked do cells arrest in the G1 phase of the cell cycle with a single chromosome (Domian et al., 1997). Although the ClpXP protease has been shown to mediate CtrA proteolysis (Jenal and Fuchs, 1998), the mechanisms ensuring that degradation occurs only at the correct place and time in the cell cycle have not been previously identified. Because both ClpX and ClpP are present throughout the cell cycle, additional factors are expected to direct the proteolysis of CtrA. CtrA proteolysis must be turned off later in the cell cycle to allow for CtrA accumulation, as active CtrA is essential at later times in the cell cycle to prevent additional rounds of DNA replication and for transcriptional activation of many cell functions.

One clue to the mechanism controlling CtrA degradation is that CtrA appears at the incipient stalked pole at the time of its degradation, and this polar localization is linked to CtrA proteolysis (Ryan et al., 2002, 2004).

Here, we show that the ClpX and ClpP subunits of the ClpXP protease dynamically localize to the incipient stalked cell pole coincident with CtrA polar localization and present biochemical evidence that CtrA interacts with ClpX in the cell, showing that CtrA degradation occurs specifically at the cell pole. In addition, we report the identification of a new cell cycle-regulated protein, conserved among the α-proteobacteria, that localizes in an identical manner to ClpX and ClpP and is required for the specific degradation and polar localization of CtrA. This *r*egulator of *C*trA *d*egradation (RcdA) protein forms a complex with both CtrA and ClpX in the cell. Thus, the dynamic polar positioning of the ClpXP protease together with the cytoplasmic RcdA protein mediates CtrA proteolysis at the appropriate time in the cell cycle.

RESULTS

Identification of RcdA

A candidate gene that contributes to the degradation of CtrA at a specific time in the cell cycle was identified in the course of seeking additional CtrA-regulatory genes. We approached this by mining previously published genomic data sets.

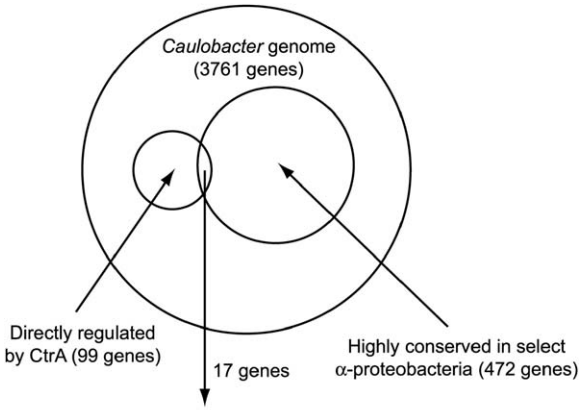
Seven cell cycle-regulated proteins—the DivJ, PleC, and CckA histidine kinases, the response regulators DivK and CtrA, the DNA methyltransferase CcrM, and the GcrA global regulator that oscillates out of phase with CtrA during the cell cycle—regulate at least one of the four redundant mechanisms that control CtrA activity (Domian et al., 1999; Holtzendorff et al., 2004; Hung and Shapiro, 2002; Jacobs et al., 1999; Reisenauer and Shapiro, 2002; Ryan et al., 2004). Transcription of four of these seven genes, *ctrA*, *gcrA*, *ccrM*, and *divK*, is also directly regulated by CtrA. CtrA directly regulates the transcription of only 3% of the entire genome, and therefore CtrA-regulated genes are overrepresented (3% versus 57%) among the set of genes that regulate CtrA activity. Thus, direct regulation by CtrA is a possible indicator to identify additional genes that regulate CtrA activity. Further, these seven genes are all highly conserved in nine α-proteobacteria (listed in Experimental Procedures). Thus, to find candidate factors involved in the regulation of CtrA activity, we identified proteins that are (1) directly regulated by CtrA and (2) highly conserved among the nine α-proteobacteria mentioned above (see Table S2 in the Supplemental Data available with this article online for the list of bacterial genome sequences used in this analysis). The seventeen genes encoding proteins that fit these two criteria are shown in Figure 1. Two of these genes, CC1035 and CC3295, encoded proteins of unknown function and were investigated further. Deletion of CC1035 did not yield an identifiable phenotype. However, CC3295, now named *rcdA* (for *r*egulator of *C*trA *d*egradation), proved to be involved in CtrA proteolysis as described below.

Characterization of *rcdA*

The genomic neighbors of *rcdA* (CC3295) are shown in Figure 2A. CC3294, encoding a conserved protein of unknown function, ends 182 bp upstream of the predicted *rcdA* start codon. The nearest downstream gene, CC3296, encoding another conserved protein of unknown function, is 11 bases downstream of *rcdA* but on the opposite strand. This genomic organization suggests that *rcdA* is transcribed as a single gene.

We constructed an *rcdA* deletion/insertion mutation (Δ*rcdA::hyg*) by replacing all but the first and last 36 bp of the *rcdA* coding region with a hygromycin-resistance cassette (Blondelet-Rouault et al., 1997). The growth rate (data not shown) and morphology of a strain (LS4185) harboring the mutation were similar to that of the wild-type with the exception that stalks were about twice as long as wild-type stalks. The *rcdA* mutant cells were sensitive to phages ΦCbK and ΦCr30, indicating the presence of pili and an S layer (Edwards and Smit, 1991; Skerker and Shapiro, 2000). Cells of the *rcdA* mutant did not form diffuse colonies on swarm agar; however, they were observed to be motile under the microscope. In a laboratory strain of *Caulobacter crescentus*, NA1000 (Evinger and Agabian, 1977), swarmer cells have a higher density than stalked and predivisional cells and can be separated in a density gradient. Density gradient centrifugation of *rcdA* mutant cells yielded only 20% as many high-density swarmer cells compared to NA1000 cultures, suggesting a low-density swarmer phenotype. Longer stalks, failure to form swarm colonies, and low-density swarmer phenotypes have all been observed previously in strains with mutations in cell cycle regulatory genes.

A polyclonal antibody raised against RcdA$(His)_6$ was used to assay RcdA levels as a function of the cell cycle. Immunoblots of lysates from wild-type and *rcdA* mutant (LS4185)



Gene	Protein Product
CC0049	S-adenosylmethionine methyltransferase
CC0050	S-adenosylmethionine synthetase
CC0378	CcrM
CC0484	Peptidyl-tRNA hydrolase
CC1035	Unknown function
CC1902	LexA
CC1923	Ribosomal protein S2
CC1963	ClpP
CC2245	GcrA
CC2463	DivK
CC2540	FtsZ
CC2551	MurG
CC3035	CtrA
CC3201	Ribosomal protein S7
CC3202	Ribosomal protein S12
CC3295	Unknown function (RcdA)
CC3475	SigT

Figure 1. Identification of CtrA-Regulating Genes

Here we show a schematic of the set of criteria used to find candidate genes that regulate CtrA stability, phosphorylation state, transcription, or localization. By considering previously known regulators of CtrA activity, two criteria were chosen to identify possible additional regulators of CtrA activity—direct regulation by CtrA and conservation (as determined by blastp) in nine other α-proteobacteria (listed in Experimental Procedures). Seventeen of the 3761 *Caulobacter* genes met both of these criteria and are listed below the diagram. Fifteen of these genes were of known function. The CC1035 knockout strain did not have an observable phenotype. CC3295 (*rcdA*) proved to be required for the ClpXP-mediated degradation and localization of the CtrA master regulator.

cells showed that the RcdA(His)$_6$ antibody recognized a single protein in wild-type cells that was not present in the *rcdA* mutant strain (Figure 2B) and that migrated in SDS-PAGE at the predicted size of RcdA. Therefore, we conclude that the antibody recognizes the native RcdA protein.

We isolated swarmer cells (Evinger and Agabian, 1977) and allowed them to proceed synchronously through the cell cycle, collecting samples every 20 min. We analyzed cell lysates by SDS-PAGE and probed the immunoblots with antibodies against RcdA and CtrA (Figure 2C). RcdA and CtrA levels were quantified using ImageQuant software (Molecular Dynamics) and graphed as a function of the cell cycle. CtrA levels varied normally over the cell cycle and served as a control for the synchrony (Domian et al., 1997). Levels of RcdA were low in newly isolated swarmer cells and peaked at the onset of the swarmer-to-stalked cell transition, coincident with the proteolysis of CtrA.

RcdA Is Necessary for the Specific Proteolysis of CtrA

CtrA is degraded in stalked cells at the swarmer-to-stalked cell transition and in the nascent stalked cell compartment of the late predivisional cell (see Figure 4A). To determine if RcdA affects the regulated proteolysis of CtrA, we performed immunoblots with anti-CtrA antibodies on samples taken from synchronized populations of wild-type and *rcdA* mutant cells at 20 min intervals over the course of the cell cycle (Figure 3A). The wild-type strain exhibited normal loss of CtrA during the G1-S transition. In the *rcdA* mutant strain, however, the levels of CtrA remained nearly constant throughout the cell cycle, suggesting that RcdA is required for the temporally regulated degradation of CtrA. To verify that the constant levels of CtrA observed in the *rcdA* mutant strain were not due to asynchronous growth, we probed the same Western blots with antibody against the McpA chemoreceptor which is also normally degraded at the G1-S transition (Alley et al., 1993). In contrast to CtrA levels, McpA levels in the *rcdA* mutant strain were indistinguishable from wild-type, indicating that RcdA is not required for temporal control of McpA degradation (Figure 3A). This result confirms that the *rcdA* mutant strain proceeded through the synchrony in a similar manner to wild-type cells and also has important implications for the function of RcdA. McpA proteolysis is similar to CtrA proteolysis in three respects: (1) McpA is degraded at the G1-S transition (Alley et al., 1993); (2) McpA is degraded by the ClpXP protease (Potocka et al., 2002); and (3) the essential response regulator DivK is required for the temporally controlled degradation of both McpA and CtrA (Hung and Shapiro, 2002). The normal degradation of McpA observed in *rcdA* mutant cells precludes a general effect of RcdA on either DivK or ClpXP functions.

To confirm that proteolysis of CtrA was affected by the absence of RcdA, pulse-chase experiments were performed to compare the half-life of CtrA conditionally expressed in wild-type and *rcdA* mutant cells (Figure 3B). The results show that the half-life of CtrA in cells lacking RcdA is significantly longer than its half-life in wild-type cells.

Two redundant mechanisms are used by *Caulobacter* cells to eliminate activated CtrA from the cell so that the G1-S transition can proceed. Regulated proteolysis is one mechanism; the second is regulation of the CtrA phosphorylation state. Mutant *Caulobacter* strains with stable CtrA remain viable owing to regulation of CtrA phosphorylation (Domian et al., 1997). Consequently, since RcdA is necessary for the regulated proteolysis of CtrA, we predicted that the regulated phosphorylation of CtrA would be essential to the G1-S transition in the *rcdA* mutant strain. To test this hypothesis, we constructed a strain that both lacks RcdA and contains a CtrA variant that cannot be regulated by dephosphorylation (Figure 3C). This variant of CtrA, CtrAD51E, contains an aspartate-to-glutamate mutation that mimics the function of the phosphorylated aspartate in wild-type CtrA.

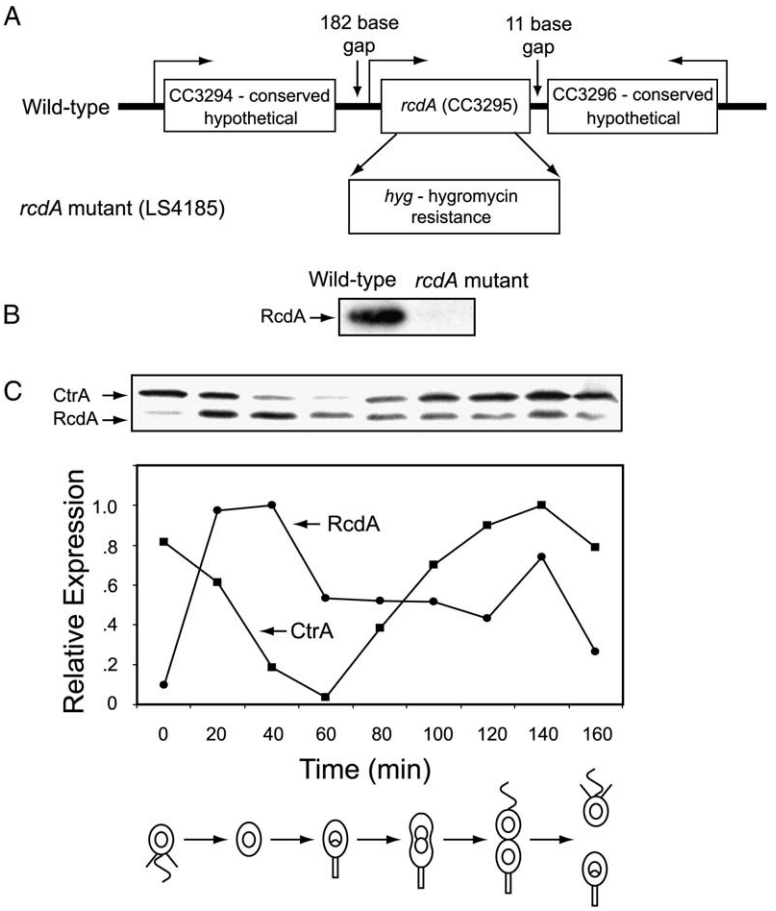


Figure 2. The RcdA Protein Is Cell Cycle Regulated

(A) Genomic organization around *rcdA*. A deletion/insertion strain (LS4185) was generated by replacing the majority of *rcdA* with a hygromycin resistance cassette.

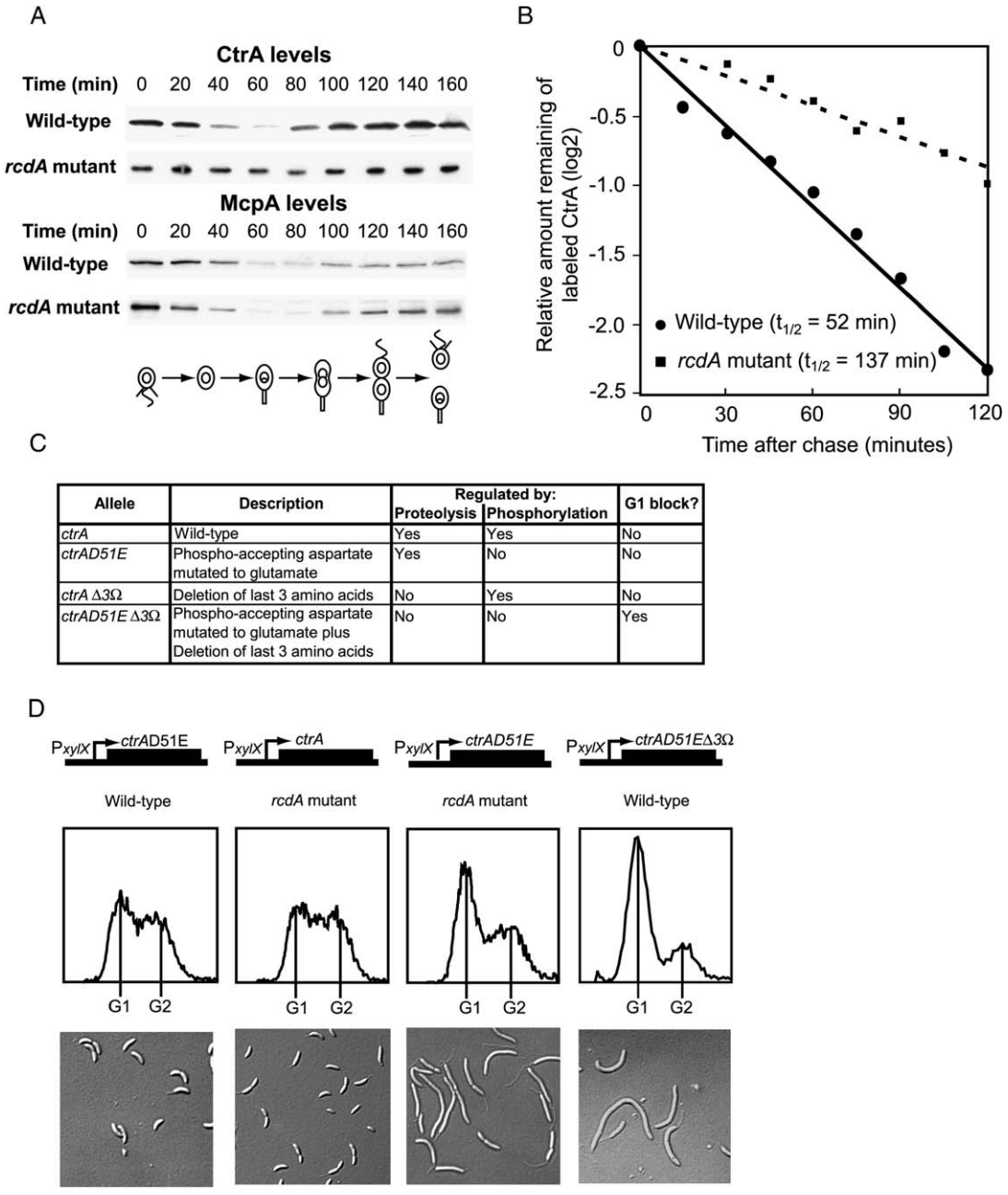
(B) Polyclonal antibody raised against RcdA(His)$_6$ recognizes one protein migrating at the predicted size of RcdA (19 kDa). This protein is not present in the *rcdA* mutant strain.

(C) RcdA and CtrA protein levels were monitored using immunoblots on cell lysates taken from samples of synchronized cultures as they proceeded through the cell cycle. RcdA protein levels peak at the time CtrA is being degraded during the swarmer-to-stalked cell transition.

In a wild-type background, CtrAD51E activity is only regulated by proteolysis; therefore, if RcdA mediates the degradation of CtrAD51E, active CtrA should be maintained at all times in the *rcdA* mutant strain, generating a G1 cell cycle arrest (Figure 3C). We conditionally expressed *ctrAD51E* for 8 hr in *rcdA* mutant and wild-type cells (Figure 3D). Consistent with previous reports, CtrAD51E had no effect on morphology or the G1-S transition when expressed in wild-type cells. However, when *ctrAD51E* was expressed in the *rcdA* mutant strain, most cells were elongated and contained only one chromosome by FACS analysis, indicating that the CtrAD51E protein remains active in the absence of RcdA and blocks the cell cycle in G1. Conditional expression of just the wild-type CtrA protein in *rcdA* mutant had no observable effect on cell morphology or chromosome content, confirming that the G1 arrest results from the loss of regulation of both the phosphorylation state and CtrA proteolysis. This result is identical to the effect of *ctrAD51EΔ3Ω* allele on wild-type cells (Figure 3D): CtrAD51EΔ3Ω is identical to CtrAD51E, except it contains an additional mutation that blocks CtrA proteolysis (Domian et al., 1997). Therefore, the loss of RcdA phenocopies a mutation in CtrA that prevents its proteolysis. Considering all these observations, we conclude that RcdA is required for the temporally regulated proteolysis of CtrA.

RcdA Is Dynamically Localized during the Cell Cycle

To determine the cellular position of RcdA, we integrated a plasmid into the *rcdA* chromosomal locus that encodes a translational fusion of *egfp* to the last 300 bp (out of ~500 bp encoding *rcdA*) of *rcdA*. This resulting strain contains a chromosomally encoded full-length version of *rcdA* fused to *egfp* under the control of the *rcdA* promoter, followed by the vector backbone and the last 300 bp of the *rcdA* gene. Immunoblots against lysates from this strain using anti-RcdA antibody revealed the presence of a single band running at the predicted size of the RcdA-GFP fusion protein. CtrA is degraded normally in this strain, indicating that the RcdA-GFP fusion protein is functional (data not shown). Using this strain, we followed the subcellular localization of RcdA by observing a synchronized population during the cell cycle with time-lapse fluorescence microscopy (Figure 4B). RcdA-GFP moved first to the incipient stalked pole during the G1-S transition and to the stalked pole in late predivisional cells, coincident with the observed localization of CtrA. RcdA-GFP also transiently localized to the division plane of late predivisional cells in about 25% of the cells. Localization of RcdA-GFP to the division plane generally preceded its localization to the stalked pole, although simultaneous localization to the midcell and stalked pole was observed in about 5% of the cells.



Allele	Description	Regulated by: Proteolysis	Regulated by: Phosphorylation	G1 block?
ctrA	Wild-type	Yes	Yes	No
ctrAD51E	Phospho-accepting aspartate mutated to glutamate	Yes	No	No
ctrA Δ3Ω	Deletion of last 3 amino acids	No	Yes	No
ctrAD51E Δ3Ω	Phospho-accepting aspartate mutated to glutamate plus Deletion of last 3 amino acids	No	No	Yes

Figure 3. RcdA Is Required for the Regulated Proteolysis of CtrA

(A) Western blot showing that the CtrA level is not cell cycle regulated in the *rcdA* deletion/insertion strain (LS4185). Immunoblots were made using samples taken at the indicated points in the synchronized cell cycles from wild-type and *rcdA* mutant cultures grown in minimal medium. Abundance of the McpA chemoreceptor, another protein degraded during the swarmer-to-stalked cell transition, is indistinguishable in wild-type and *rcdA* mutant cells, showing that RcdA is not needed for McpA proteolysis.

(B) Pulse-chase assay shows that the CtrA average half-life is 2.6 times longer in *rcdA* mutant cells compared to wild-type (unsynchronized cultures).

(C) Activated CtrA is redundantly regulated by proteolysis and phosphorylation. The *ctrAD51E* allele is effectively permanently activated. The CtrAΔ3Ω protein is not degraded by ClpXP. The *ctrAD51E*Δ3Ω allele is effectively permanently activated and not degraded. The strain containing *ctrAD51E*Δ3Ω exhibits a G1 block, whereas the other strains and the wild-type do not.

(D) Constitutive CtrA activation by the replacement of aspartate with glutamate causes G1 arrest in the *rcdA* deletion/insertion strain. The CtrA variants identified in (C) were expressed in wild-type and *rcdA* mutant cells for eight hours before DIC microscopy and FACS analysis. Expression of *ctrAD51E*, which mimics the phosphorylated state of CtrA, in wild-type cells had minimal effect on cell shape and DNA content. Expression of this *ctrAD51E* in the *rcdA* mutant, however, yielded elongated cells containing one chromosome, indicating a G1 cell cycle arrest. This combination phenocopies expression in wild-type cells of *ctrAD51E*Δ3Ω, which contains mutations that both mimic CtrA phosphorylation and prevent regulated CtrA degradation.

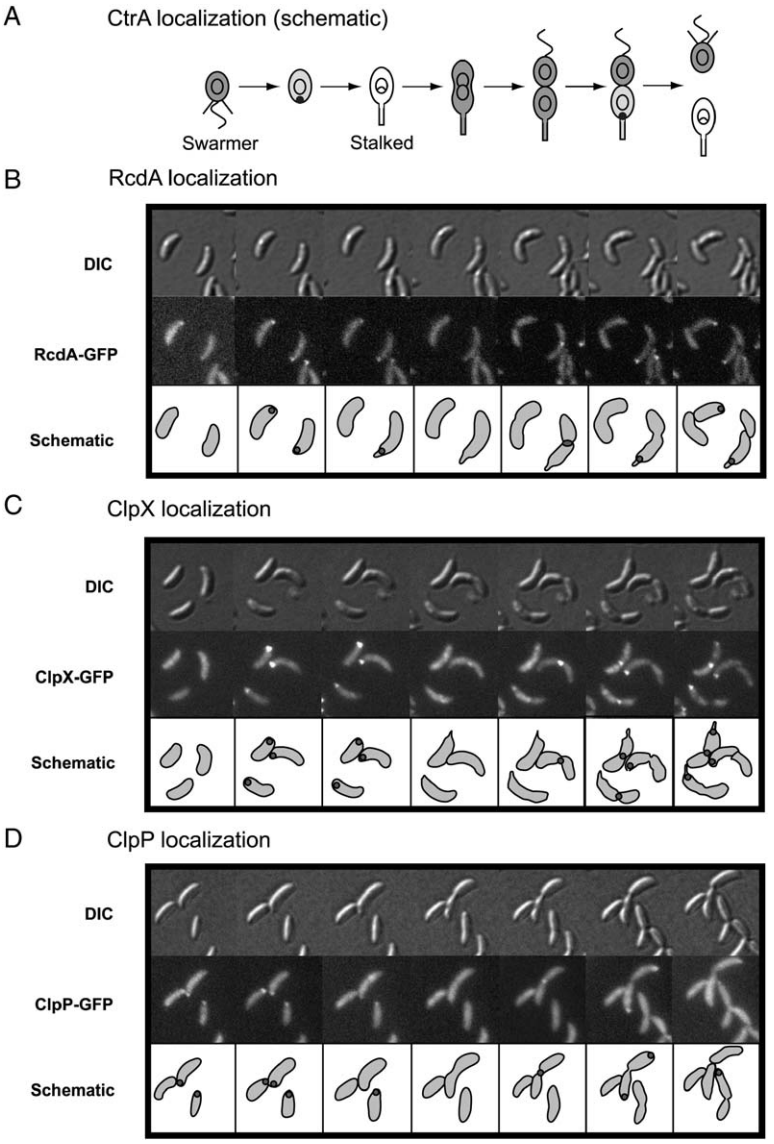


Figure 4. Dynamic Localization of Proteins Involved in the Regulated Degradation of CtrA

(A) Schematic of CtrA localization during the cell cycle. CtrA is cleared from the cell during the swarmer-to-stalked cell transition and from the stalked compartment of the late predivisional cell after cytoplasmic compartmentalization (Domian et al., 1997; Judd et al., 2003). Localization of CtrA to the cell pole at the indicated times is linked to its proteolysis (Ryan et al., 2004).

(B) RcdA-GFP localizes to the stalked pole at the swarmer-to-stalked cell transition, transiently to the division plane in late predivisional cells, and then to the stalked pole of the daughter stalked cell. RcdA-GFP was expressed from the *rcdA* promoter as the only copy of *rcdA*. Swarmer cells were harvested, resuspended on an agarose pad containing minimal media, and thereafter imaged by DIC and fluorescence microscopy every 45 min.

(C) The ClpX ATPase component of the ClpXP protease parallels the localization pattern of RcdA. Cells containing a xylose-inducible *clpX-egfp* were grown in minimal media and incubated for 1 hr with xylose before harvesting swarmer cells. Isolated swarmer cells were resuspended on an agarose pad containing minimal medium with xylose and thereafter imaged by DIC (upper panel) and fluorescence microscopy (middle panel) every 45 min. A schematic of these cells is shown in the lower panel.

(D) The localization pattern of the ClpP protease component of the ClpXP protease parallels the pattern of RcdA in (B) and the ClpX component in (C). Cells containing a xylose-inducible *clpP-egfp* were grown in PYE media and incubated for 1 hr with xylose before harvesting swarmer cells. Isolated swarmer cells were resuspended on an agarose pad containing PYE media with xylose and thereafter imaged by DIC (upper panel) and fluorescence microscopy (middle panel) every 30 min.

ClpX and ClpP Dynamically Localize to the Cell Pole as a Function of the Cell Cycle

The ClpXP protease, which is present throughout the cell cycle, is responsible for the degradation of CtrA at the swarmer-to-stalked cell transition and in the nascent stalked cell compartment of the late predivisional cell (Jenal and Fuchs, 1998). We previously showed that YFP-tagged CtrA appears as a point focus at the incipient stalked pole coincident with its proteolysis (Ryan et al., 2002; Figure 4A), and here we show that the RcdA protein is localized at the same time and place as CtrA. To determine if the ClpXP protease colocalizes with CtrA and RcdA at the cell pole, we examined the cellular position of both components of the ClpXP protease, ClpX and ClpP, as a function of the cell cycle (Figures 4C and 4D).

To observe the dynamic intracellular position of the ClpXP protease, we created merodiploid strains with either *clpX-egfp* or *clpP-egfp* expressed from an inducible chromosomal locus. We integrated a construct encoding a translational fusion of the C terminus of *clpX* or *clpP* to *egfp* into the chromosomal *xylX* locus of wild-type cells. These strains were grown in minimal (ClpX-GFP) or PYE (ClpP-GFP) media. After induction with xylose for one hour, we performed time-lapse microscopy on synchronized cells to determine the subcellular location of ClpX-GFP or ClpP-GFP at different points during the cell cycle (Figures 4C and 4D). We found that the subcellular localization of ClpX-GFP and ClpP-GFP is identical. Both appeared as a single focus at the incipient stalked cell pole, coincident with the time of CtrA polar localization at both the swarmer-to-stalked cell transition and also in the nascent stalked cell compartment of the late predivisional cell. ClpX-GFP and ClpP-GFP also transiently localized to the cell division plane in late predivisional cells just before their localization to the stalked pole of the predivisional

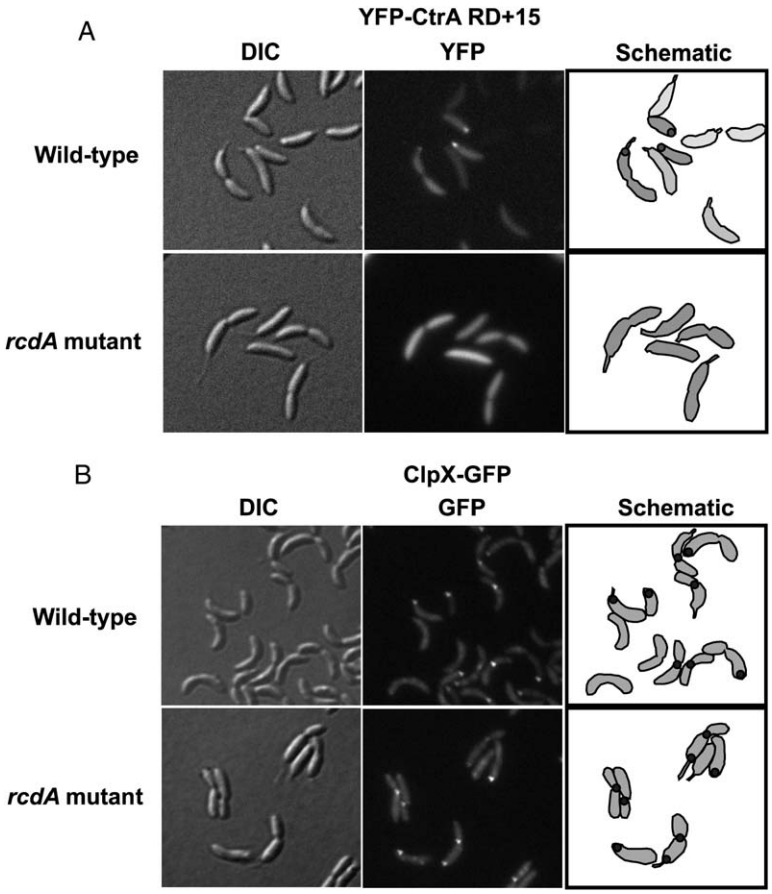


Figure 5. RcdA Is Required for CtrA's Transient Localization to the Cell Pole but Is Dispensable for ClpX Localization

(A) RcdA is required for CtrA localization. Wild-type or *rcdA* mutant cells containing a plasmid encoding *yfp-ctrA RD+15* under control of the inducible xylose promoter were treated for 1 hr with xylose and observed using DIC and fluorescence microscopy. In wild-type cells (upper image), YFP-CtrA RD+15 formed polar foci at the stalked pole during the swarmer-to-stalked cell transition and in the stalked compartment of late predivisional cells. In *rcdA* mutants (lower image), YFP-CtrA RD+15 no longer formed foci and was dispersed throughout the cytoplasm.

(B) RcdA is dispensable for ClpX localization. Wild-type or *rcdA* mutant cells containing *clpX-egfp* integrated at the *xylX* locus were treated for 1 hr with xylose to induce expression of *clpX-egfp* and observed using DIC and fluorescence microscopy. In both backgrounds, ClpX-GFP localizes to the stalked pole in cells undergoing the swarmer-to-stalked cell transition and late predivisional cells, as well as to the division plane in late predivisional cells.

cell. The localization of ClpX, ClpP, and RcdA to the division plane suggests that these proteins may be involved in another degradation pathway specific to proteins involved in cell division. In swarmer cells and in early predivisional cells, both proteins were dispersed throughout the cytoplasm. While ClpP-GFP localized to the same cellular positions as ClpX-GFP, more of the ClpP-GFP protein was cytoplasmic as compared to the ClpX-GFP protein. This result could be explained if ClpX protein recruited ClpP to the cell pole. Quantification the protein levels of ClpX and ClpP revealed there are two ClpP tetradecamers for each ClpX hexamer (Østerås et al., 1999), and therefore there is not enough ClpX in the cell to recruit all of the ClpP protein to the pole. The additional cytoplasmic localization of ClpP compared to ClpX could also be a result of ClpP's interaction with the ClpA ATPase or other uncharacterized ATPase components.

RcdA Is Necessary for CtrA Localization, but Not for ClpX Localization

Since both CtrA localization and proteolysis require the same 56 amino acid sequence in the CtrA receiver domain, we asked whether RcdA, which is necessary for CtrA degradation, is also required for CtrA localization to the cell pole. To address this question, we examined the cellular position of YFP-CtrA RD+15 (Ryan et al., 2002) in an *rcdA* mutant background strain. YFP-CtrA RD+15 is an N-terminal YFP fusion to the receiver domain and last 15 amino acids of CtrA, which contains the minimal requirements for cell cycle-regulated degradation and polar localization of CtrA (Ryan et al., 2002). In *rcdA* mutant cells, CtrA foci were not present in stalked or late predivisional cells, and diffuse fluorescence was observed in all cell types (Figure 5A). This diffuse fluorescence is likely not an artifact of higher levels of YFP-CtrA RD+15, as polarly localized CtrA has been previously observed using a nondegradable allele of CtrA with mutations in the two most C-terminal amino acids (Ryan et al., 2002). Thus, RcdA is required for both the degradation and localization of CtrA, suggesting RcdA mediates CtrA recruitment to the cell pole.

To determine if loss of RcdA also affects the dynamic localization of ClpX, we expressed *clpX-egfp* from the *xylX* locus in the *rcdA* mutant (Figure 5B). After inducing expression of *clpX-egfp* with xylose, ClpX-GFP still formed polar foci at the incipient stalked pole during the swarmer-to-stalked cell transition and at the division plane and stalked pole in late predivisional cells. Therefore, the proper positioning of ClpX at the cell pole and the division plane is independent of the polar location of either RcdA or CtrA.

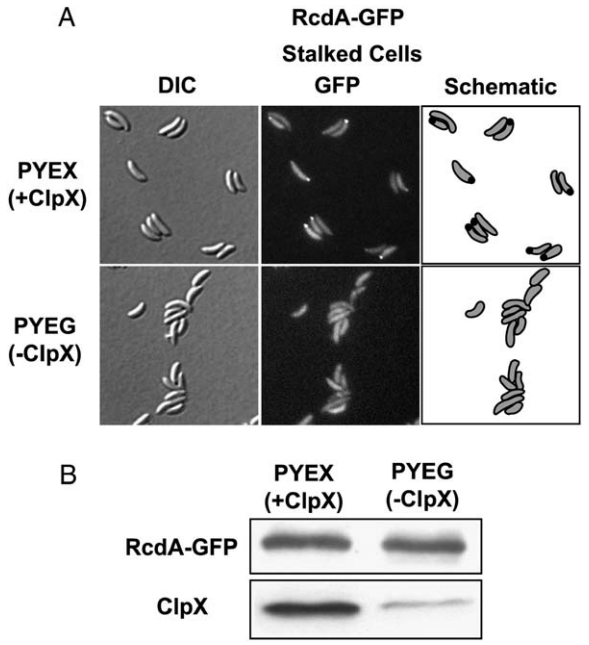


Figure 6. ClpX Is Required for Localization of RcdA to the Cell Pole

(A) *rcdA-egfp* expressed from the *rcdA* promoter as the only copy of *rcdA* in the cell was introduced into an inducible ClpX background. Cells were grown for 6 hr in PYEX (+ClpX) and PYEG (−ClpX) media. By this time, most of the ClpX protein was depleted from the culture grown in PYEG (B). Cells were synchronized from both cultures and allowed to proceed through the cell cycle in the same media they had been grown in. At 20 min, cells were observed using DIC and fluorescence microscopy. RcdA-GFP localized to the cell pole in cells grown in permissive media (PYEX). The culture grown in PYEG, depleted ClpX, did not correctly localize RcdA-GFP to the cell pole.

(B) Immunoblots of samples taken concurrent with the fluorescent microscopy. Both cultures have similar amounts of RcdA-GFP. The culture grown in restrictive media (PYEG) had significantly reduced amounts of ClpX.

ClpX Is Required for RcdA Localization to the Cell Pole

The above results show that RcdA is necessary for the localization of CtrA. However, the resolution of light microscopy does not allow us to distinguish whether RcdA is recruited to the localized ClpXP protease or to another polar site. ClpX is essential in *Caulobacter* (Jenal and Fuchs, 1998), so we could not address this question using a deletion of *clpX*. Instead, we used a previously described ClpX depletion strain that contains *clpX* under the control of the inducible *xylX* promoter as the only copy of *clpX* on the chromosome (Jenal and Fuchs, 1998). We introduced into the strain a chromosomally encoded fusion of *egfp* to the C terminus of *rcdA* under the control of the *rcdA* promoter. The resulting strain (LS4195) allowed us to follow the localization of RcdA-GFP in cells depleted of ClpX (Figure 6A).

The LS4195 strain was grown in the presence of xylose (PYEX) before shifting half of the culture to glucose-containing medium (PYEG) for 6 hr. At this time, most of the ClpX protein was depleted from the cells grown in PYEG (Figure 6B). We isolated swarmer cells from both the PYEG and PYEX cultures, resuspended them in PYEG and PYEX, respectively, and allowed them to proceed through the cell cycle. As previously reported (Jenal and Fuchs, 1998), under these conditions cells in both cultures grew and formed stalks, but most of the cells grown in PYEG (depleted of ClpX) did not form a constriction at the division plane. Using fluorescence microscopy, we followed RcdA-GFP localization in both cultures. In the culture grown in PYEX, RcdA-GFP localized in the same pattern as in wild-type background (58% of cells had foci, 236 cells counted); in contrast, in the PYEG culture which was depleted of ClpX, the RcdA-GFP no longer formed a single focus at the cell pole during the swarmer-to-stalked cell transition (8% of cells had foci; 188 cells counted; Figure 6A). Immunoblots of lysate taken from cells grown in both PYEG and PYEX confirmed that RcdA-GFP was still present in the PYEG-grown culture (Figure 6B). Since the cells grown in PYEG had a cell division defect, we could not draw conclusions regarding the dependence of RcdA's localization to the division plane on ClpX.

Thus, we conclude that RcdA is necessary for CtrA localization to the cell pole and that RcdA localization is dependent on ClpX. Furthermore, neither RcdA nor CtrA is required for the polar position of ClpX. Based on these observations, we propose that RcdA is required for interaction of CtrA and the polarly localized ClpXP protease.

ClpX, RcdA, and CtrA Interact In Vivo

The colocalization of CtrA, RcdA, and ClpX to the incipient stalk pole during the swarmer-to-stalked cell transition and to the stalked pole in late predivisional cells, as well as their genetic dependencies for polar localization, suggest that these proteins physically interact to achieve the degradation of CtrA. To address this, we performed coimmunoprecipitation experiments. We created three strains, LS4326, LS4328, and LS4327, with the *ctrA*, *clpX*, or *rcdA* gene translationally fused to an M2-epitope tag at the respective locus, resulting in a replacement of the wild-type gene with a C-terminal, M2-tagged allele.

To determine if the RcdA protein interacts with the CtrA-M2 or ClpX-M2 protein in vivo, we added M2 monoclonal antibody attached to agarose beads to cell lysates taken from LS4326 (CtrA-M2) or LS4328 (ClpX-M2) strains. The agarose beads were centrifuged, washed, and collected. Coimmunoprecipitated proteins were eluted, separated by gel electrophoresis, and transferred to a membrane. Immunoblots were performed on each of these samples using CtrA or ClpX antibody to verify that the CtrA-M2 or ClpX-M2 protein was efficiently immunoprecipitated (data not shown). Immunoblots using antibody to RcdA were then carried out to determine if RcdA coimmunoprecipitated with the CtrA-M2 or ClpX-M2 protein (Figure 7A). The RcdA antibody recognized a protein that comigrates with RcdA in M2 immunoprecipitates of the LS4326 and LS4328 samples (Figure 7A, lanes 2 and 3). This protein was not present in the wild-type sample treated with M2 antibody agarose beads but was present in whole-cell extracts (Figure 7A, lane 1),

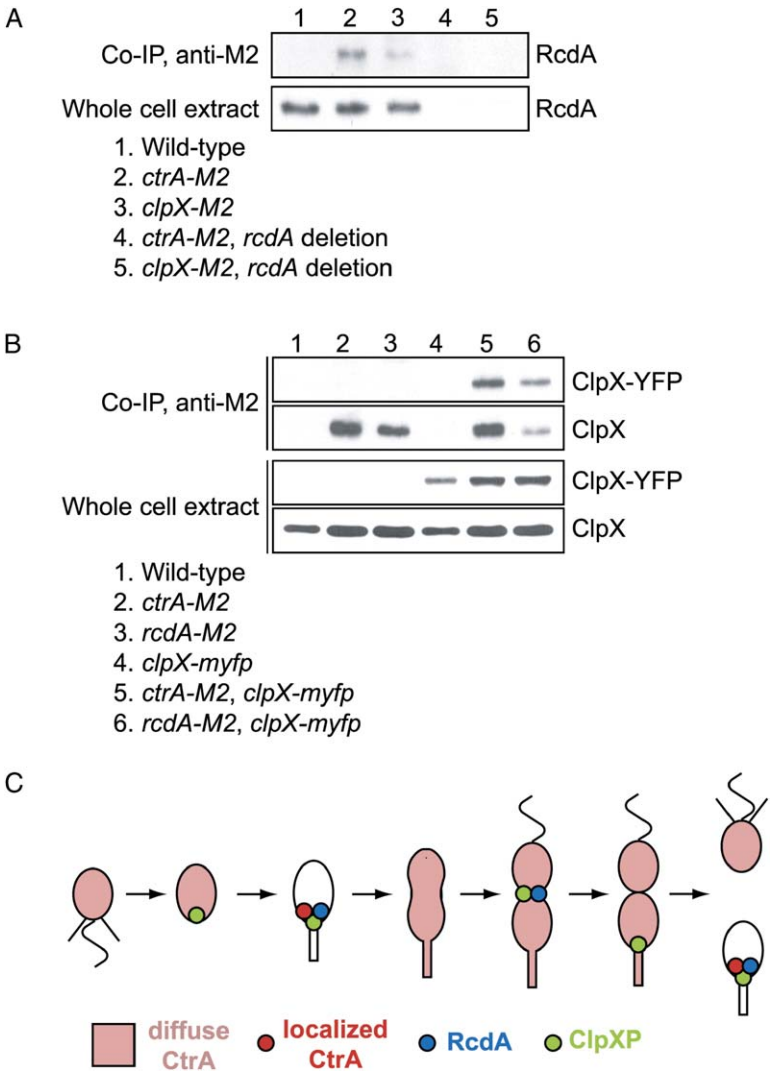


Figure 7. ClpX, RcdA, and CtrA Interact In Vivo

(A) Monoclonal antibody recognizing the M2-epitope tag was used to immunoprecipitate M2-tagged CtrA or M2-tagged ClpX protein from cell lysates bearing a replacement of wild-type *ctrA* or *clpX* with the M2-tagged allele. Immunoblots of these samples using antibody to RcdA show that a protein comigrating with RcdA is present in immunoprecipitates of both CtrA-M2 and ClpX-M2 (lanes 2 and 3) but is not present in immunoprecipitates from an *rcdA* mutant background (lanes 4 and 5). Although RcdA is present in immunoblots of wild-type cells, it cannot be detected in samples of wild-type cell extracts immunoprecipitated with antibody to the M2 tag (lane 1). This provides biochemical evidence that RcdA interacts with CtrA-M2 and ClpX-M2 in the cell.

(B) Antibody recognizing the M2-epitope tag was used to immunoprecipitate M2-tagged CtrA or RcdA from cell lysates bearing a replacement of wild-type *ctrA* or *rcdA* with an M2-tagged allele. Immunoblots using ClpX antibody revealed a protein comigrating with ClpX that is present in immunoprecipitates of both CtrA-M2 and RcdA-M2 (lanes 2 and 3). This protein was not present in samples taken from wild-type cells (lane 1), indicating this protein interacts with CtrA-M2 and RcdA-M2 protein, and is not recognized by the M2 antibody. To verify that this protein is ClpX, we immunoprecipitated M2-tagged CtrA and RcdA from strains that also expressed a ClpX-mYFP protein fusion. In these strains, an additional protein coimmunoprecipitated with CtrA-M2 and RcdA-M2 that ran at the predicted size of ClpX-mYFP protein (lanes 5 and 6). Thus, ClpX and ClpX-mYFP interact with CtrA-M2 and RcdA-M2 protein in the cell.

(C) Diagram of the dynamic localization of RcdA, ClpXP, and CtrA as a function of the cell cycle. At the swarmer-stalked cell transition and in the stalked compartment of the late predivisional cell, ClpXP localizes to the cell pole through a mechanism independent of RcdA and CtrA. RcdA and CtrA then colocalize to the polar ClpXP protease, and RcdA is required for both CtrA polar localization and proteolysis. As the cell cycle progresses, CtrA is degraded, the ClpXP protease and RcdA leave the pole, and then cytoplasmic CtrA reaccumulates. Both ClpXP and RcdA transiently appear at the division plane and then at the pole of the incipient stalked cell compartment, where CtrA is again localized followed by its proteolysis.

indicating that the protein specifically coimmunoprecipitates with CtrA-M2 or ClpX-M2 and does not associate with the M2 antibody. To verify that the protein coimmunoprecipitating with ClpX-M2 and CtrA-M2 is RcdA, we repeated these experiments on lysates of two strains (LS4329 and LS4330) identical to LS4326 or LS4328 with the exception that the majority of the *rcdA* gene is replaced by a hygromycin-resistance cassette. Immunoblots with RcdA antibody showed that the coimmunoprecipitating protein was no longer present in either LS4329 or LS4330 (Figure 7A, lanes 4 and 5), verifying that the protein coimmunoprecipitating with CtrA-M2 and ClpX-M2 is RcdA (Figure 7A).

To confirm these results, coimmunoprecipitating experiments were designed to demonstrate that ClpX interacts with the CtrA-M2 and RcdA-M2 protein. CtrA-M2 or RcdA-M2 was immunoprecipitated from LS4326 or LS4327, respectively, using the M2 antibody bound to agarose beads. Immunoblots using ClpX antibody recognized a protein in these immunoprecipitates that comigrated with ClpX (Figure 7B, lanes 2 and 3). This protein was not present in the samples taken from the wild-type lysate (Figure 7B, lane 1), indicating that the protein coimmunoprecipitates with CtrA-M2 and RcdA-M2 and is not recognized by the M2 antibody. To verify that this protein is ClpX, we repeated these experiments on cell lysates taken from three new strains (LS4331, LS4332, and LS4333). Each of these CB15N-derived strains contain a plasmid encoding a xylose-inducible *clpX-myfp* translational fusion (*myfp* encodes a monomeric version of the YFP protein) integrated at the chromosomal *xylX* promoter. The ClpX-mYFP protein fusion

was induced in each of these strains by the addition of xylose. LS4332 and LS4333 also include a replacement of the wild-type *ctrA* or *rcdA* gene with a plasmid encoding a M2-tagged allele of *ctrA* or *rcdA*, respectively. Immunoblot analysis with the ClpX antibody again revealed a protein identical in size to ClpX that coimmunoprecipitates with either CtrA-M2 or RcdA-M2 protein (Figure 7B, lanes 4, 5, and 6). An additional band was now found that ran at the predicted size of the ClpX-mYFP protein fusion. This band was not found in the samples taken from the LS4331 lysate, indicating that this higher molecular weight protein coimmunoprecipitates with CtrA-M2 and RcdA-M2 protein and does not associate with M2 antibody. These data provide biochemical evidence that ClpX, CtrA, and RcdA form a complex within the cell.

DISCUSSION

We have examined the temporally controlled degradation of the *Caulobacter* master regulator CtrA to probe the mechanism of regulated proteolysis in bacteria and to understand its role in the genetic circuitry controlling the bacterial cell cycle. The timing of CtrA proteolysis is critical for *Caulobacter* cell cycle progression. CtrA is degraded by the ClpXP protease, yet ClpX and ClpP are present throughout the cell cycle (Jenal and Fuchs, 1998). Therefore, additional factors must operate to control the timing of CtrA proteolysis. We have shown here that temporal control of CtrA degradation is mediated by the combined actions of dynamic ClpXP positioning in the cell and a newly identified protein, RcdA.

The ClpXP Protease Is Dynamically Localized during the Cell Cycle

We previously showed that the cytoplasmic CtrA protein is recruited to the stalked cell pole coincident with its degradation at the G1-S transition and also to the pole of the nascent stalked compartment of the late predivisional cell (Ryan et al., 2004). Analysis of *ctrA* mutant strains and *ctrA* homologs from other α-proteobacteria showed that CtrA polar localization can occur without proteolysis, but not the reverse, suggesting that CtrA localization is mechanistically upstream of degradation (Ryan et al., 2004). We now find that both components of the ClpXP protease are also dynamically localized to the cell pole coincident with CtrA localization and degradation. Thus, the process of CtrA degradation is spatially restricted to a protease that is itself physically constrained to a polar position within the cell. This occurs at the time in the cell cycle when clearance of active CtrA is needed to advance the cell cycle program.

The transmembrane chemoreceptor McpA is also degraded at the swarmer-to-stalked transition by ClpXP (Tsai and Alley, 2001), and McpA is also located at the incipient stalked pole at the time of its proteolysis (Alley et al., 1992). Many other putative *Caulobacter* chemoreceptors localize to the cell pole (M.R.K. Alley, personal communication), and they also contain the conserved peptide motif that is necessary for the degradation of McpA (Alley, 2001; Alley et al., 1993; Tsai and Alley, 2001). This suggests that several ClpXP substrates are located at the incipient stalk cell pole during the swarmer-to-stalked transition. The polar localization of both CtrA and McpA just at the time of their degradation by polar-localized ClpXP suggests that the ClpXP substrates must be colocated with the localized protease, or in its immediate vicinity, to be degraded. We propose that the dynamic localization of ClpXP enables ClpXP-mediated proteolysis of a specific set of proteins.

In addition to its transient localization to two polar sites, ClpXP is transiently localized to the division plane (Figure 7C). Identification of ClpXP substrates at each localization site will be critical to understanding ClpXP's overall role in cell cycle progression. *Caulobacter* proteins, FtsZ and FtsA, are localized to the division plane (Din et al., 1998; Martin et al., 2004), and they are degraded coincident with the presence there of ClpXP (Kelly et al., 1998; Martin et al., 2004). The proteolysis of these two proteins, as well as other components of the divisome, may also be mediated by the localized ClpXP protease. Additionally, recent work in *B. subtilis* has shown that ClpX, but not ClpP, inhibits the formation of the FtsZ ring (Weart et al., 2005). Physical interaction between ClpX and FtsZ has also been observed in *E. coli* (Flynn et al., 2003), suggesting ClpX serves as a regulator of FtsZ ring formation in many different bacteria. The timing of localization of ClpX to the division plane is consistent with such a role in *Caulobacter*. The colocalization of ClpP and RcdA to the division plane, however, suggests that there is an additional proteolytic role for ClpXP and RcdA at the division plane. The deletion of *rcdA* results in altered cell morphology (longer stalks, small swarms on swarmer plates, and a cell-density defect). It is likely that RcdA is required for the proteolysis of additional cell cycle-controlled proteins.

A Novel Protein, RcdA, Is Required for CtrA Proteolysis and Colocalizes with CtrA at the Polar ClpXP Protease

The initiation of CtrA proteolysis requires its localization to the cell pole and its specific interaction with ClpXP protease at the pole. Through a bioinformatics search for CtrA regulators, we have identified a cell cycle-regulated gene, *rcdA*, whose expression is required for the localization and proteolysis of CtrA. Using coimmunoprecipitation experiments, we have provided biochemical evidence that RcdA, CtrA, and ClpX are part of a complex within the cell. At the swarmer-to-stalked cell transition RcdA appears to be specific for CtrA, since RcdA is dispensable for the ClpXP-mediated degradation of the polar McpA chemoreceptor. How might RcdA mediate the degradation of CtrA? Our finding that RcdA interacts with CtrA and ClpX likely precludes a signal transduction role for RcdA. However, RcdA is not absolutely required for an interaction between ClpX and CtrA, as ClpX can still coimmunoprecipitate with CtrA in the absence of RcdA (A.A.I., unpublished data). This interaction is not functional, as CtrA is not degraded in an *rcdA* mutant strain (Figure 3). RcdA is required for the localization of CtrA to the polar ClpX (Figure 5), indicating RcdA is required for the interaction of CtrA with ClpX at the cell pole but dispensable for the nonfunctional interaction between CtrA and nonpolar

ClpX. We believe the most likely role for RcdA is in mediating interaction of CtrA with the polar ClpXP complex. In this model, CtrA can interact with the non-polarly localized ClpX in the absence of RcdA, but only when RcdA is present and interacting with both proteins can CtrA localize to the cell pole, allowing the CtrA/ClpX interaction to be functional for degradation. A consequence of this model is that CtrA must be at the cell pole in order to be degraded, as has been previously shown (Ryan et al., 2004). Because RcdA also colocalizes with ClpXP at the division plane, RcdA may be a required factor for the proteolysis of other proteins at the time and site of cell division.

The regulation of *rcdA* is another component of the overall regulation of CtrA degradation. There is a CtrA binding site upstream of *rcdA*, and gene expression microarray experiments on a strain bearing a temperature-sensitive *ctrA* allele suggested that CtrA turns on *rcdA* expression (Laub et al., 2002). RcdA protein levels dramatically increase during the swarmer-to-stalked cell transition just before CtrA is degraded, suggesting that upregulation of *rcdA* expression is necessary for subsequent CtrA degradation. This mutual coregulation pattern, where CtrA regulates a protein that also regulates CtrA, is not unique to RcdA: CtrA regulates the transcription of three other genes—*gcrA*, *divK*, and *ccrM*—whose products regulate CtrA activity. Each of these cases involves negative feedback where the protein product of a gene that is positively regulated by CtrA has a negative effect on either CtrA activity or the level of CtrA in the cell or vice-versa.

A Phosphotransfer Signaling Pathway Influences the Timing of CtrA Proteolysis

CtrA is a major node in the control of the *Caulobacter* cell cycle, and a phosphotransfer signal pathway operates to redundantly control CtrA activity at many different levels. Localized degradation of CtrA by ClpXP represents only one piece in the complex puzzle of CtrA regulation. DivK, an essential single-domain response regulator, is required for both the degradation and polar localization of CtrA, as well as for the specific degradation of the McpA chemoreceptor during the swarmer-to-stalked cell transition (Hung and Shapiro, 2002; Ryan et al., 2004). The histidine kinases PleC and DivJ have also been shown play a role in the spatially asymmetric degradation of CtrA in compartmentalized cells nearing cell division (Judd et al., 2003; Ryan et al., 2004). PleC and DivJ may influence CtrA proteolysis through DivK, since DivJ phosphorylates DivK in vivo, while the histidine kinase PleC acts primarily as a phosphatase of DivK (Matroule et al., 2004). Altogether, these observations suggest that a signaling pathway involving DivK, DivJ, and PleC contributes to the regulation of CtrA degradation. The essential histidine kinase CckA, a protein that regulates CtrA's phosphorylation state (Jacobs et al., 1999), also affects the stability of CtrA (Jacobs et al., 2003), either via the DivK pathway or a separate pathway. Signaling using these phosphotransfer proteins is likely to determine when in the cell cycle CtrA is degraded. Determining how the localization of ClpXP and regulation of RcdA are influenced by this signaling cascade will be critical to understanding regulation of the bacterial cell cycle.

EXPERIMENTAL PROCEDURES

Identification of Candidate Regulators of CtrA Activity

We initially identified a small set of genes that are (1) directly regulated by CtrA and (2) highly conserved among the nine α-proteobacteria: *Agrobacterium tumefaciens*, *Bartonella henselae*, *Bartonella quintana*, *Bradyrhizobium japonicum*, *Brucella melitensis*, *Brucella suis*, *Mesorhizobium loti*, *Rhodopseudomonas palustris*, and *Sinorhizobium meliloti*. We identified genes directly regulated by CtrA based on biochemical analysis (Holtzendorff et al., 2004; Hung and Shapiro, 2002) or results of ChIP/Chip experiments reported in Laub et al. (2002). Genes conserved among the nine α-proteobacteria were identified using the blastall 2.2.10 software (Altschul et al., 1990). For each *Caulobacter* gene, the top blast hit from each bacterial sequence available in August, 2004 with an E value less than 10^{-10} was recorded and ordered by bit score. We selected genes whose top nine hits were from the nine α-proteobacteria listed above.

Microscopy

For time lapse microscopy, cells were applied to a 1% agarose pad containing M2G media, M2G media supplemented with 0.3% xylose, or PYE media supplemented with 0.3% xylose. For all other experiments, cells were resuspended on a pad containing 1% agarose in water. DIC and fluorescence images were acquired as previously described (Ryan et al., 2002). Fluorescein (Chroma 96170M), and YFP (Chroma 41028) filter sets were used to acquire GFP and YFP images, respectively. Fluorescent exposures were for 2–5 s.

Coimmunoprecipitation Experiments

Cells were grown in 500 ml of PYE (0.3% xylose was added when appropriate) until reaching an OD_{660} of 0.3. Cultures were centrifuged for 20 min at 8000 rpm at 4ºC, and the pellet was washed in CoIP buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, and 20% glycerol), and centrifuged again in the same conditions. Pellets were frozen at −80ºC until future use. For analysis, pellets were melted on ice, washed with CoIP buffer, and resuspended in 5 ml of CoIP buffer with 0.05% Triton X-100. Samples were incubated with 10 mM $MgCl_2$, 50 mg of lysozyme, and 50 units of DNase I (Fermentas) at 4ºC for 30 min, and then lysed three times with a French press at 16,000 psi. The lysates were centrifuged at 13,000 rpm at 4ºC for 5 min and the supernatants were collected. Supernatants were incubated with 20 μl of anti-FLAG M2-agarose affinity gel (FLAGIPT-1, Sigma) at 4ºC overnight. Samples were then pelleted, washed three times with CoIP buffer, and then washed three times with wash buffer (50 mM Tris-HCl [pH 7.4] and 150 mM NaCl). After the last wash, the supernatant was removed, and the pellets were resuspended with 150 μl of wash buffer containing FLAG peptide (100 ng/μl; Sigma) and incubated for 1 hr at 4ºC. Samples were then centrifuged at 13,000 rpm for 2 min to pellet the agarose beads. Supernatants were collected and frozen at −20ºC until immunoblot experiments were performed, as described above.

Strains, Plasmids, Growth Conditions, Antibody Production, FACS Experiments, Immunoblots, and Half-Life Experiments

Detailed descriptions are provided in the Supplemental Data. See Table S1 for a list of strains and plasmids.

Supplemental Data

Supplemental Data include two tables and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/124/3/535/DC1/.

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